A Single Amino Acid Substitution Confers Progesterone 6β-Hydroxylase Activity to Rabbit Cytochrome P450 2C3*

(Received for publication, October 20, 1992)

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The complete nucleotide sequence of the cDNA encoding 2C3 has been derived from a partial cDNA and gene sequences for 2C3 (9, 10). Characterization of the cognate protein expressed in COS-1 cells or in Escherichia coli indicates that it encodes the 6β-form (11). Based on the high degree of structural similarity expected for the two forms of 2C3, we reasoned that a PCR-based approach could be utilized to isolate and identify a cDNA encoding the 6β-form of 2C3 (23v) by using primers corresponding to the reported sequence of the 2C3 gene (9) and first strand cDNAs prepared from New Zealand White rabbit liver RNA. In this report, we describe the successful isolation of a cDNA encoding 2C3v that catalyzes progesterone 6β-hydroxylation and identify five differences in its predicted amino acid sequence relative to residues that affect changes in the catalytic properties of P450s align at a position which maps to a substrate-contacting surface loop in the bacterial enzyme P450cam (4). The region containing these key amino acid residues is highly variable among closely related P450 enzymes which have distinct catalytic functions. We have proposed a framework model for P450 enzymes in which substrate-contacting surface loops readily accommodate genetic changes that lead to changes in substrate specificity without altering the basic topological organization of the enzymes (3–5). Other regions are more highly conserved and may be part of basic topological features, such as the heme binding site that is required for the reduction of oxygen. Based on this model, we have demonstrated that we could transfer a hypervariable domain from P450 2C5 to P450 2C1, two enzymes that share less than 75% amino acid identity, and confer a new catalytic activity, progesterone 21 hydroxylation, to 2C1 (3).

The characterization of naturally occurring variants of P450 has been especially useful for identifying critical amino acids for substrate specificity and enzymatic activity. We have characterized the biochemical properties of closely related rabbit liver P450 enzymes that catalyze 6β- and 16α-hydroxylation of progesterone (6). Only one of these forms, designated as 6β*, catalyzes the 6β-hydroxylation of progesterone, whereas the other, designated as 6β, does not (6). Both forms catalyze 16α-hydroxylation, but the 6β-form exhibits a higher catalytic efficiency for this activity. In addition, 16α-methylprogesterone selectively inhibits both the 6β- and 16α-hydroxylation activities catalyzed by the 6β form, whereas it slightly stimulates the 16α-hydroxylase activity of the 6β-form. This activation of the 6β-form is more apparent for 5β-pregnane-3β,20α-diol, a naturally occurring catabolic product of progesterone (7). Preparations of 2C3 isolated from outbred New Zealand White or inbred III/J rabbit liver appear to be a mixture of both the 6β- and 5β-forms (6, 8). In contrast, preparations of 2C3 isolated from inbred III/J rabbit liver do not contain the 6β-form (6, 8).

The abbreviations and conventions used are: P450, cytochrome P450; TLC, thin-layer chromatography; PCR, polymerase chain reaction; SRS, substrate recognition site; diol, 5β-pregnane-3β,20α-diol. Mutations are indicated using the one-letter abbreviation for the amino acid residue that was replaced, its position in the sequence, and the one-letter designation of the new residue in the indicated order.

*This work was supported by United States Public Health Service Grant GM31001 (to E. F. J.) and GM35897 (to B. K.). Facilities for computer-assisted analysis and the synthesis of oligonucleotides are supported in part by General Clinical Research Center Grant RR00953 and by the Sam and Rose Stein Charitable Trust, respectively. The costs of publication of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2C3. Expression of chimeric constructs from 2C3 and 2C3v in COS-1 cells and in E. coli revealed the amino acid difference at residue 364 is the determinant of the 6β-hydroxylation of progesterone. Sequence alignments (12, 13) predict that this amino acid position corresponds to a region that forms a portion of the substrate binding site within the polypeptide chain of P450acom.

MATERIALS AND METHODS

Production of P450 2C3 cDNAs by PCR—The synthesis of cDNA from total RNA prepared from a New Zealand White rabbit liver (14) and its subsequent amplification by PCR using the GeneAmp PCR Kit followed the method described by Perkin-Elmer Cetus. Briefly, first strand cDNAs were prepared with MuLV reverse transcriptase (GIBCO/BRL) using either random 9-mers (Stratagene) or oligo(dT) (Boehringer Mannheim) as primers. Double-stranded cDNAs were then synthesized by PCR using Tag DNA polymerase and two oligonucleotide primers which correspond to the 5' and 3' ends of the coding region of 2C3 based on the published sequence (9, 10). The upstream primer, 5'-GAAGATCGCCATGGATCTCCTCATTA- TCCATCCTG-3' corresponds to the 5' end of the coding strand (nucleotides -11 to +21) and contains an additional BglII site at its 5' end (underlined). The downstream primer, 5'-GCTCTAGATCA- GACTGGGAAACAAAGACCTCA-3', corresponds to the 3' end of the complementary strand (nucleotides 1478-1510) and contains an additional XbaI site which is underlined. The PCR reactions utilized an Eppendorf thermal cycler and 35 repetitions of the following cycle: 94°C 1 min (denature), 55°C 1 min (anneal), 72°C 2 min (extend) followed by a single incubation for 7 min at 72°C. The approximately 1.5-kilobase pair PCR products were isolated from 1% agarose gels (Seakem GTG grade, FMC BioProducts) and purified using the GeneClean Kit (Bio101). Purified PCR products were digested with BglII and XbaI and ligated into the expression vector, pCMV (15), using T4 DNA ligase (Bethesda Research Laboratories). The ligated DNAs were used to transform competent E. coli DH5α (Bethesda Research Laboratories). Initial analysis utilized DNA purified by a modification of the method employing hexadecyl trimethyl ammonium bromide (16). The nucleotide sequences of the longest representative SpeI+ and BglII and XbaI and ligated into the expression vector, pCMV (15), using T4 DNA ligase (Bethesda Research Laboratories). The ligated DNAs were used to transform competent E. coli DH5α (Bethesda Research Laboratories). Initial analysis utilized DNA purified by a modification of the method employing hexadecyl trimethyl ammonium bromide (16).

Cloning of 2C3 cDNAs from a Rabbit Liver Library—A probe, CAGTTGTGTCAGAGATTC, corresponding to nucleotides 302-321 of the 2C3 cDNA, was synthesized and used to screen a rabbit liver cDNA library provided by Dr. D. Russell of the University of Texas Southwestern Medical School and used with the permission of Dr. M. Stinski, University of Iowa, was employed for the expression of 2C3-related proteins from their respective cDNAs in COS-1 cells (ATCC). DNA for transfection was prepared by the alkaline lysis method followed by a CaCl2 centrifugation (14). COS-1 cells were cultured and transiently transfected as described previously (3). The 6β- and 16α-hydroxylation of progesterone was determined at 72 h after transfection by supplementation of the culture medium for 2 h with 10 μM [4-14C]progesterone (57.2 Ci/mmol, Du Pont-New England Nuclear), followed by extraction and analysis by thin layer chromatography (TLC) on silica gel (5). Metabolites and substrate were separated by TLC as described (19), with one modification to improve resolution. Extracted substrate and products were first separated using benzene:methyl acetate (3:1) prior to the use of two solvent systems employed in earlier work (6). Metabolites were quantified by liquid scintillation counting.

Sequence Analysis—Nucleotide sequencing utilized the dideoxy-nucleotide chain termination method (20) with [γ-32P]ATTPs (>1000 Ci/mmol, Amersham Corp.) and T7 DNA polymerase based sequencing Kits (Pharmacia LKB Biotechnology Inc. and United States Biochemical Corp.). Fourteen oligonucleotide primers corresponding to the 2C3 cDNA were utilized to verify the complete sequence on both strands of all constructs. Sequencing gels consisted of 6% acrylamide and 7.8 M urea. The electrophoresis buffer was 1× TBE (14). In some cases, the gel was run for 1 h with 0.5× TBE in the TBE buffer chamber, 1× TBE in the TAE buffer chamber and then the buffer in the lower chamber was altered by addition of 0.5 volume of 3 M sodium acetate, pH 5.0, to increase the number of readable bases per reaction (21).

Heterologous Expression of 2C3 Enzymes in E. coli—The pCW expression vector was obtained from Dr. R. Dahlquist (Institute of Agricultural Biology, University of Oregon, E. coli). These plasmids were used to transform the E. coli strain XL-1 Blue (Stratagene) which served as the expression host. The nucleotide sequences of the longest representative SpeI and SpeI cDNAs were determined.

Construction of Chimeric and Site-directed Mutagenesis—Chimeric constructs were made by joining the 5' and 3' coding regions of 2C3 and 2C3v in pCMV by exchanging restriction fragments, generated using the restriction enzymes: BstBI, MscI, and PpuMI. The resulting constructs were verified by complete sequence analysis. The S364T mutation in 2C3 (2C3:S364T) was generated using a two-step PCR procedure for site-directed mutagenesis developed by Landt et al. (18). The first PCR reaction utilized as primers the aforementioned upstream primer (nucleotides -11 to +21) and a specific mutagenic oligonucleotide, CATTGGGGCAAGATGATGG, which is complementary to nucleotides 1083-1102 of the 2C3 cDNA. The underlined nucleotide indicated the mutation introduced into 2C3. The first PCR product consisted of 30 cycles of 1 min at 94°C, 1 min at 45°C and 2 min at 72°C, followed by a 7-min incubation at 72°C. The gel-purified PCR product and the downstream primer described above (nucleotides 1478-1510) were used as the primers for a second PCR reaction. The second PCR reaction consisted of 35 cycles as described above for the amplification of the 2C3 cDNAs. Both PCR reactions utilized the 2C3 cDNA as template and Vent DNA polymerase (New England Biolabs). The final PCR product was isolated from a 1% agarose gel, purified with the Geneclean Kit (Biolabs). The final PCR product was isolated from a 1% agarose gel, purified with the Geneclean Kit (Biolabs). The final PCR product was isolated from a 1% agarose gel, purified with the Geneclean Kit (Biolabs). The final PCR product was isolated from a 1% agarose gel, purified with the Geneclean Kit (Biolabs).

Expression in COS-1 Cells—The expression vector, pCMV5, obtained from Dr. D. Russell of the University of Texas Southwestern Medical School and used with the permission of Dr. M. Stinski, University of Iowa, was employed for the expression of 2C3-related proteins from their respective cDNAs in COS-1 cells (ATCC). DNA for transfection was prepared by the alkaline lysis method followed by a CaCl2 centrifugation (14). COS-1 cells were cultured and transiently transfected as described previously (3). The 6β- and 16α-hydroxylation of progesterone was determined at 72 h after transfection by supplementation of the culture medium for 2 h with 10 μM [4-14C]progesterone (57.2 Ci/mmol, Du Pont-New England Nuclear), followed by extraction and analysis by thin layer chromatography (TLC) on silica gel (5). Metabolites and substrate were separated by TLC as described (19), with one modification to improve resolution. Extracted substrate and products were first separated using benzene:methyl acetate (3:1) prior to the use of two solvent systems employed in earlier work (6). Metabolites were quantified by liquid scintillation counting.

Isolation of cDNA Encoding the 6β Form of 2C3—In order to isolate and characterize the 6β variant of 2C3, PCR primers were designed, based on the sequence of the gene (9) encoding the 6β form of 2C3, which would yield a complete coding region and contained restriction sites for unidirectional insertion into the pCMV5 vector for the subsequent transfection of COS-1 cells. First strand cDNAs were generated from total liver RNA obtained from an outbred rabbit and served as templates for the polymerase chain reaction. The products were expected to include both the 6β and 6β forms of 2C3.
The PCR products were isolated, ligated into pCMV, and 22 transformants of E. coli were chosen for preliminary characterization by limited restriction mapping using BamHI, BspHI, EcoRI, Nhel, PvuMI, Smal, and SpeI. Interestingly, a SpeI site was not present in four of the 22 clones, suggesting that they might encode a variant of 2C3. When the four SpeI-clones were expressed in COS-1 cells, progesterone was hydroxylated at both the 6β- and 16α-positions (Fig. 1), indicating that these cDNAs encoded the 6β* form of 2C3. In contrast, cells transfected with the SpeI+ clones exhibited only 16α-hydroxylation activity as expected for 2C3 (11). If cells transfected with the latter clones exhibited 6β-/16α-hydroxylase activity ratios similar to that of the SpeI- clones, 6β-hydroxylase activity would have been readily detected in these experiments based on the levels of 16α-hydroxylation detected for cultures transfected with the SpeI+ clones. Thus, the SpeI+ and SpeI- clones reflect variants of 2C3 which differ in their capacity to catalyze the 6β-hydroxylation of progesterone.

Complete sequence analysis of three SpeI+ and three SpeI- PCR-derived cDNAs revealed six nucleotide differences between 2C3 (SpeI*) and 2C3v (SpeI-) which resulted in five amino acid differences and one silent mutation (Table I). The serine/threonine difference at position 364 reflects a single nucleotide difference that is responsible for the loss of the SpeI site in 2C3v. All six nucleotide differences were observed in three completely sequenced SpeI- cDNAs obtained from three independent PCR reactions, whereas the SpeI+ cDNAs corresponded to 2C3. We sought to confirm the natural occurrence of all six of these nucleotide differences in partial cDNAs obtained from an independent rabbit liver cDNA library which had been generated without PCR amplification. SpeI digestion was used to distinguish the partial cDNAs corresponding to either 2C3v or 2C3. Of the four clones examined, one was found to be SpeI+. A representative clone for each cDNA was completely sequenced. All six of the nucleotide differences observed between the PCR-derived 2C3 and 2C3v cDNAs were also found to exist in the corresponding cDNAs obtained from the rabbit liver cDNA library. No additional differences between the SpeI+ and SpeI- cDNAs were noted in the 3'-untranslated regions.

Heterologous Expression of 2C3v—Although the expression of the 2C3v in COS cells established that the enzyme catalyzes the 6β-hydroxylation of progesterone, it is difficult to determine the concentrations of P450 enzymes expressed in COS-1 cells and, thus, to precisely define the turnover number of the enzyme. Expression of these enzymes in E. coli, and their subsequent isolation and characterization provides a means for a more complete determination of their enzymic properties for comparison to preparations of P450 3b from rabbit liver microsomes. In addition, larger amounts of enzyme can be obtained more economically to facilitate this characterization. For this purpose, the N-terminal coding sequence of the 2C3v cDNA was modified to facilitate heterologous expression in E. coli as described earlier for 2C3 (11). The 2C3v-encoded protein expressed in E. coli was purified and reconstituted with reductase. The enzyme exhibits an apparent Km of 1.2 μM and a Vmax of 5.6 μM/min/μM P450 for the 6β-hydroxylation of progesterone and a Km of 1.4 μM and a Vmax of 2.0 μM/min/μM P450 for the 16α-hydroxylation of progesterone. Preparations of 2C3 from rabbits that contain both the 6β* and 6β- forms of 2C3 exhibit a Km estimated to be less than 1 μM, with a Vmax in the range of 1–3 μM/min/μM P450 for 6β-hydroxylation of progesterone (6, 8). Kinetic parameters for the high-efficiency 16α-hydroxylase activity catalyzed by 6β* preparations of P450 3b purified from rabbit liver had been estimated by subtraction of the component arising from the low efficiency 6β- enzyme to yield values for Km of 0.3 μM and for Vmax of 0.5 μM/min/μM P450.

The inhibitor, 16α-methylprogesterone, was found to inhibit the 6β- and 16α-hydroxylation catalyzed by reconstituted 2C3v (Fig. 2). These results mirror those found for preparations of P450 2C3 from rabbit liver that contain the 6β* form and support the conclusion that 2C3v encodes the form of 2C3 catalyzing progesterone 6β-hydroxylation.

When 10 μM 5β-pregnane-3β,20α-diol was included in the reconstitution assay, the efficiency of the 16α-hydroxylation catalyzed by 2C3v was increased reflecting a lower Km for progesterone and a higher Vmax. A similar effect of 5β-pregnane-3β,20α-diol on the 6β-hydroxylase activity of 2C3v was also observed (Fig. 2), although this effect was relatively small and had not been reported previously for preparations of P450 2C3 from rabbit liver that contain the 6β* form (8).

T364S Is Necessary for the 6β-Hydroxylation of Progesterone by 2C3v—Chimeric cDNAs were constructed by exchanging restriction fragments between 2C3 and 2C3v in the mammalian expression vector pCMV5 in order to identify which of the amino acid differences between 2C3 and 2C3v determine the ability of 2C3v to catalyze 6β-hydroxylation. Expression in transfected COS-1 cells combined with an in vivo

![Fig. 1. Progesterone metabolism by COS-1 cells transfected with PCR-derived P450 2C3 cDNAs. 2C3 cDNAs could be separated into two groups based on the presence or absence of a SpeI restriction site. COS-1 Cells were transfected with the pCMV5 vector alone (Mock) or the vector containing the SpeI+ or SpeI- 2C3 cDNAs. Forty eight hours after transfection, the medium was removed and replaced by medium containing 10 μM [14-C]progesterone. After a 2-h incubation at 37°C, the medium was removed and analyzed. An autoradiogram of the thin layer chromatogram for one example of the metabolites, 6β-hydroxyprogesterone (6β-OH-P) and 16α-hydroxyprogesterone (16α-OH-P), is indicated at the right.](image-url)

#### Table I

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<th>Position</th>
<th>2C3</th>
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<td>Codon</td>
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<td>476</td>
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progestosterone assay was used to qualitatively assess the ability of each chimeric protein to catalyze progestosterone 6β-hydroxylation. These constructs and their capacity to catalyze the 6β-hydroxylation of progesterone are summarized in Fig. 3. Hybrids in which N- or C-terminal regions of 2C3 (containing changes at positions 178 and 256 or 472 and 476, respectively) were replaced with those of 2C3v did not confer 6β-hydroxylase activity. In contrast, substituting a larger C-terminal fragment of 2C3v for that of 2C3v, including the S364T change, did confer the 6β-hydroxylase activity. These results indicate that the capacity to catalyze 6β-hydroxylation is dependent on the presence of a threonine residue at position 364 rather than a serine. To confirm this, the reciprocal point mutations were made in 2C3 and 2C3v. The single mutation, S364T, in 2C3 confers 6β-hydroxylase activity to 2C3, whereas the reciprocal mutation in 2C3v deletes this activity.

The two single-mutant proteins, 2C3:S364T and 2C3v:T364S, were expressed in E. coli with a modified N-terminal membrane anchor domain (11) and purified for detailed kinetic characterization following reconstitution with P450 reductase. The Ser/Thr difference at position 364 not only determines the enzyme's capacity for 6β-OH, but it is also a determinant for selective inhibition by 16α-methylprogestosterone. For the single mutants, this inhibitor was found to inhibit progesterone 6β- and 16α-hydroxylations catalyzed by 2C3:S364T as is seen for 2C3v, but under the same conditions, it did not inhibit the 16α-hydroxylation catalyzed by the 2C3v:T364S as is observed for 2C3v (not shown). 2C3v:S364T was also seen to exhibit similar values of Vmax for the 6β- and 16α-hydroxylase activities as 2C3v. However, two results indicate the involvement of other amino acids in the kinetic differences observed between 2C3 and 2C3v. The Km values for progesterone observed for 2C3v:S364T were about 8-fold higher for 16α-hydroxylation and 2-fold higher for 6β-hydroxylation than the respective Km values obtained from 2C3v (Table II). Moreover, the 2C3v:T364S mutant exhibited a higher catalytic activity than 2C3v over the range of substrate concentrations examined (Fig. 4). These results suggest that one or more of the four remaining amino acid differences influence the relative values of apparent Kmax for progesterone exhibited by the two enzymes.

Additional Amino Acid Differences Contribute to the Distinct Enzymic Properties of 2C3 and 2C3v—In order to identify which of the four remaining amino acid differences contribute to differences between the single mutants and the parental enzymes in their apparent Kmax for progesterone, additional chimeras were constructed and heterologously expressed in E. coli. Characterization of these chimeras indicated that the I178M difference contributes to differences in the apparent Kmax for progesterone between 2C3v, 2C3v, and the single mutants in which residue 364 is exchanged. As shown in Fig. 4, the mutation I178M increases the catalytic efficiency of 2C3v, and this single mutant (2C3v:I178M) exhibits kinetic properties similar to that of the 2C3v:T364S mutant. Introduction of the M178I mutation into 2C3v:T364S converts the latter into an enzyme that is similar to 2C3 (Fig. 4). The reciprocal mutations in 2C3 (S364T and I178M) convert 2C3 into an enzyme which exhibits Vmax and Kmax values for both the 16α- and 6β-hydroxylations of progesterone that are very similar to those exhibited by 2C3v (Table II).

**DISCUSSION**

The results reported herein demonstrate that 2C3 and 2C3v are structurally highly related P450s that differ in their capacity to catalyze the 6β-hydroxylation of progesterone. The cDNA corresponding to the 6β" form (2C3v) differs at only six nucleotide positions from the reported sequence of 2C3 (9), and these nucleotide changes result in 5 amino acid differences between 2C3v and 2C3. Analysis of hybrid enzymes expressed from chimeric constructs between the 2C3 and 2C3v cDNA in COS-1 cells indicates that the S364T difference is responsible for the phenotypic difference in the expression of progesterone 6β-hydroxylase activity between 2C3 and 2C3v. However, when the kinetic properties of the 2C3v and 2C3v:S364T proteins purified from E. coli were compared, the single S364T change introduced into 2C3v did not fully mimic the enzymic characteristics of 2C3v. Although 2C3v and 2C3v:S364T each had a similar Vmax for the 6β- and 16α-hydroxylase activities, the 2C3v:S364T mutant displayed a higher apparent Kmax for both progesterone hydroxylase activities than 2C3v (Table II). This observation suggested that some or all of the other 4 amino acid differences between 2C3 and 2C3v contribute to these differences. This situation is also evident when comparing the 16α-hydroxylation activity of 2C3v:T364S with that of 2C3. 2C3v:T364S displays a higher catalytic efficiency than 2C3 but is not as efficient as 2C3v. Characterization of additional chimeric constructs indicates that the I178M mutation combined with the S364T mutation...
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When the sequence of 2C3v is compared with that of P450cam for which a three-dimensional structure is available (24), using the sequence alignments of either Laughton et al. (13) or Nelson and Strobel (12), Thr\(^{364}\) corresponds to one of the proposed substrate contact residues in P450cam, Val\(^{295}\). Atkins and Sligar (25) found that alterations of Val\(^{295}\) in P450cam to an Ile or Ala residue decreased the stoichiometry between product formation and oxygen consumption and altered the regiospecificity of product formation from camphor, 1-methylnorcamphor, and norcamphor (25). In addition, others have reported single amino acid substitutions in mammalian P450s in this region that alter catalytic activity. A L365M substitution which aligns in close proximity to T364 of 2C3v confers the coumarin hydroxylase activity of P450cam to an Ile or Ala residue decreased the stoichiometry between product formation and oxygen consumption and altered the regiospecificity of product formation from camphor, 1-methylnorcamphor, and norcamphor (25).

In contrast, the I178M difference between 2C3 and 2C3v that underlies differences between the two variants in their apparent \(K_m\) for progesterone falls outside of SRS boundaries proposed by Gotoh (26). Ile is highly conserved at this alignment position among all members of P450 families 1, 2, 3, 17, and 21 (12). A single mutation (I172N) at this alignment position in human CYP21A results in the loss of the steroid 21-hydroxylase activity that underlies some forms of adrenal hyperplasia (27, 28). It is interesting to note that a Met is found at this position in the aromatase enzyme and among family 4A P450s (12). Based on alignments with P450cam (12, 13, 26), residue 178 falls in helix E which is far from the heme-binding region and proposed substrate pocket (12, 25). We speculate that a mutation in helix E could alter its contact with helix I, resulting in a shift in the positions of the helix F-helix G loop which includes SRS-2 and SRS-3. Such a shift might contribute to the observed difference in \(K_m\).

Gotoh (26) has noted that the SRS regions of family 2 enzymes often exhibit higher frequencies of nonsynonymous substitutions when compared with other regions. In this regard, it is worth noting that five of the six nucleotide substitu-
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tutions were nonsynonymous for 2C3/2C3v and that three of the five fall in SRS regions. This suggests that selective pressure may have contributed to the occurrence of distinct enzymic forms of 2C3.

Heterologous expression of P450 enzymes at high levels and their subsequent purification, as demonstrated herein, could eventually lead to a crystal structure for the mammalian P450s. This will elucidate the mechanisms governing substrate binding as well as the inhibition and activation of P450 catalysis by negative and positive effectors. The interpretation of structural information obtained from crystallography must, necessarily, accommodate functional information. Mutagenesis studies, such as this one, should compliment crystallographic studies by providing clues to deciphering the structural characteristics which determine function.

Acknowledgments—We acknowledge Drs. H. J. Barnes, M. R. Waterman, and T. H. Richardson for their helpful suggestions concerning heterologous protein expression in E. coli. We also thank the Sam and Rose Stein trust for supporting the DNA Core Laboratory, Department of Molecular and Experimental Medicine, The Scripps Research Institute.

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